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14. ABSTRACT
The overarching objective of this work is to develop composite materials based on bacterial cellulose for military use using renewable, sustainable, and environmentally-friendly prudent processing and manufacturing techniques. Approaches to produce oriented crystalline structures to enable the formation of next-generation fibers whose properties will allow the synthesis of composites possessing improved strength and functionality will be investigated. The bacterial cellulose fibers will be produced from renewable non-food agricultural products via cultivation of bacterial species already used in food-production. The research proposed here will provide critical high-performance materials that are based on sustainable resources and green, renewable processing.A process for cellulose biosynthesis by bacteria to yield unidirectionally-oriented nanofibers formed as fibrous sheets by application of external electrical fields was devised. Approaches to synthesize novel bacterial cellulose composite materials based on task-specific ionic liquids and graphene oxide were studied. Methods to optimize the carbon yield from bacterial cellulose were also investigated.

15. SUBJECT TERMS
Bacterial cellulose, electric field alignment, carbonization, carbon-cellulose composite materials, aligned fibers

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List of Acronyms

AC: alternating current
AO: Acridine Orange
ATCC: American Type Culture Collection
BC: Bacterial Cellulose
CSD Chemical Sciences Division
DC: direct current
EDX: Energy-dispersive X-ray spectroscopy
EF: electric field
[emim][Tf2N]: 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)amide
IL: ionic liquids
IR: infra-red
MSE: Material Sciences & Engineering
ORNL: Oak Ridge National Laboratory
PI: Principal Investigator
RTIL: room temperature ionic liquid
SEM: scanning electron microscopy
SERDP: Strategic Environmental Research and Development Program
SH: Schramm & Hestrin growth medium
SMF: Son-Matsuoka-Fructose medium
UTK: University of Tennessee at Knoxville

Keywords

Bacterial cellulose, electric field alignment, carbonization, carbon-cellulose composite materials, aligned fibers

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Abstract

Objectives

The overarching objective of this work is to develop composite materials based on bacterial cellulose for military use using renewable, sustainable, and environmentally-friendly prudent processing and manufacturing techniques. Approaches to produce oriented crystalline structures to enable the formation of next-generation fibers whose properties will allow the synthesis of composites possessing improved strength and functionality will be investigated. The bacterial cellulose fibers will be produced from renewable non-food agricultural products via cultivation of bacterial species already used in food-production. The research proposed here will provide critical high-performance materials that are based on sustainable resources and green, renewable processing.

Technical Approach

A process for cellulose biosynthesis by bacteria to yield unidirectionally-oriented nanofibers formed as fibrous sheets by application of external electrical fields was devised. Approaches to synthesize novel bacterial cellulose composite materials based on task-specific ionic liquids and graphene oxide were studied. Methods to optimize the carbon yield from bacterial cellulose were also investigated.

Results

Our limited scope proposal provides critical proof-of-concept for developing new composite materials based on bacterial cellulose. Electric field alignment studies showed that the choice of media formulation, the geometry of the apparatus, and the use of planktonic (free floating cell cultures) were all important issues for cellulose synthesis in electric fields. A novel approach of templated growth of the bacteria to allow the bacteria to be propagated from a fixed position in the electric field growth chamber provided unequivocal evidence for directional synthesis of cellulose during the application of an external electric field. Two different approaches for producing bacterial cellulose composite materials were investigated. The water in bacterial cellulose hydrogels was replaced with an ionic liquid ([emim][Tf_2N]) and performed comparably to, and in some respects better than, state-of-the-art commercial porous polyethersulfone membranes in liquid membrane CO_2/N_2 separations. This novel approach marries the functional properties of ionic liquids with the structural properties of bacterial cellulose for the development of a new class of membrane. Finally, by using a dehydrating agent it was possible to obtain a near theoretical yield of carbon after pyrolysis of bacterial cellulose, which has important implications for development of carbon-based composites and high-value carbon fibers from bacterial cellulose.

Benefits

The knowledge gained in this work will be applied toward developing techniques to produce carbon fibers and other novel composite fibers with high structural strength and controlled properties. The processing methods proposed use renewable, sustainable, and environmentally responsible methods. Several patents and publications are expected to result from this work. The next step will be to produce materials with properties superior to the current state-of-the-art

petroleum-derived composite materials. Such materials will include carbon fibers and carbon cloths—militarily useful due to their low weight and high strength— as well as novel metal, ceramic, and polymer matrix composites.

Objectives

The aim of this work is to develop composite materials based on bacterial cellulose for military use using renewable, sustainable, and environmentally-friendly prudent processing and manufacturing techniques. Bacterial cellulose is synthesized as a pure, highly crystalline, polymer and is reported to have the highest Young's modulus (15–30 GPa) known amongst two dimensional organic materials. Its properties are very different from cellulose derived from plants. This material has excellent potential due to its high strength, low density, and high crystallinity. Although bacterial cellulose has many properties considered highly advantageous for the development of light weight, high strength fibers, one of the major drawbacks of using this material is that in its natural state it is produced as a tangled, randomly-oriented network of cellulose fibrils deposited in sheet-like structures (**Figure 1**). While this morphology is responsible for its high strength, the highly hydrogen bonded network is difficult to disperse into elementary single fibers without physical and chemical disruption of the cellulose and concomitant alteration of its properties.

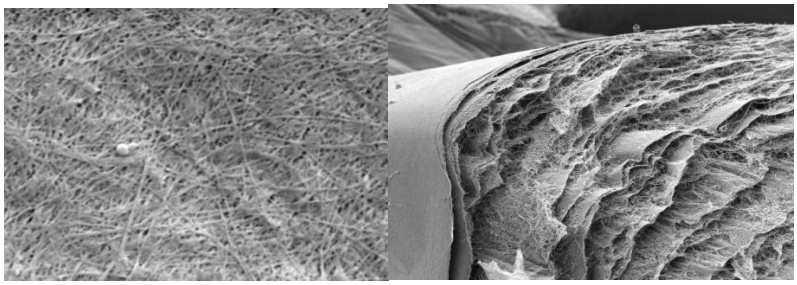

Figure 1. Scanning electron micrographs of bacterial cellulose.
Left panel: Top-down view displaying randomly-oriented cellulose fibers (viewing pane is 9 μm wide). *Right panel:* Sheet-like structure of bacterial cellulose pellicle (viewing pane is 180 μm)

The overarching objective of this work is to devise a process for *cellulose biosynthesis* that will yield unidirectionally-orientated nanofibers formed as fibrous sheets. To achieve this goal, we will investigate the application of *external electrical fields* to induce orientation of cellulose fibers as they are extruded by cellulose producing bacteria. As part of the work, the design of novel cultivation vessels and media that exploit bacterial responses to medium viscosity and air–liquid interfaces is required to further guide fiber biosynthesis. *Ionic liquids* represent a unique class of solvent that offers unprecedented versatility and tunability. Researchers have shown ionic liquids to be powerful solvents for the dissolution, processing, reconstitution, and regeneration of natural polymers like silk, wool keratin, chitin, and cellulose. In this part of the project, we will evaluate the ability of ionic liquids to solvate and disperse in bacterial cellulose for subsequent processing without disrupting the crystallinity of the material.

Approaches to produce oriented, highly regular, crystalline structures to enable the formation of next-generation fibers whose unique properties will allow the synthesis of composites possessing improved strength and functionality. The bacterial cellulose fibers will be produced

from renewable non-food agricultural products via cultivation of bacterial species already used in food-production. The research proposed here will provide critical high-performance materials that are based on sustainable resources and green, renewable processing. The knowledge gained in this work will be applied toward developing techniques to produce carbon fibers and other novel composite fibers with high structural strength and controlled properties. These high-strength, oriented, crystalline materials will also have many other applications. These include the development of sensor/actuator piezoelectric materials (smart papers) and surfaces to support guided directionally oriented regeneration and repair of fibrous tissues such as nerves and tendons.

Background

As the primary component of plant cell walls, cellulose is the most abundant natural polymer in the world with an estimated 10^{10}–10^{11} tons produced each year. Plant cellulose is associated with other biopolymers such as hemicellulose and lignin to form a laminate material. Purifying cellulose from these polymers requires harsh chemical processes, such as treatment with mineral acids, sulfur dioxide, sodium hydroxide, and bleaching agents to extract hemicellulose and lignin resulting in losses in fiber strength. Cellulose is typically regenerated using the viscose process or modified into derivatives such as cellulose acetate or carboxymethyl cellulose. In these forms, however, cellulose retains little of the crystallinity intrinsic to the native form.

Certain bacterial species, such as *Gluconacetobacter*, possess the ability to secrete pure cellulose in the form of a hydrogel, called a pellicle, with water constituting some 99.8% of the total volume of the matrix (Shoda and Sugano, 2005). Although chemically identical to plant cellulose, bacterial cellulose is morphologically distinct. When grown under static cultures in liquid media, the bacteria synthesize a highly crystalline three-dimensional cellulose network at the air–liquid interface. During this process, bacteria extrude subfibrils of pure cellulose that bundle and crystallize into nanofibrils, which then bond together to form ribbons with typical dimensions of 3–4 nm thickness, 70–80 nm width, and 1–9 μm length (Ross et al., 1991). Bacterial cellulose fibrils are up to 200 times finer than plant cellulose fibrils, producing substantial contact area and permitting a high density of inter- and intra-fibrillar hydrogen bonding which imparts the cellulose its hydrogel structure. It has been reported that dried bacterial cellulose yields the highest Young's modulus (15–30 GPa) known amongst two-dimensional organic materials (Shoda and Sugano, 2005).

Production of cellulose by *Gluconacetobacter spp*. utilizes cost-effective renewable agricultural products. Agricultural feedstocks that have been successfully used for bacterial cellulose production include corn steep liquor (Matsuoka et al., 1996) apples, beer wort (Brown, 1886; Herrmann, 1928), corn syrup, kale (black cabbage) extract, tomatoes, coconut milk (Bernardo et al., 1998), and extracts from several Brazilian plants (Fontana et al. 1990).

Previous work within our group has demonstrated that bacterial cellulose can readily be perfused sequentially with various fluids, solutions, or gases, allowing the introduction of a wide range of chemicals for formation of composite materials. Our original discovery was the spontaneous deposition of palladium nanoparticles based on reduction of hexachloropalladate ($(NH_4)_2PdCl_6$) by reaction at the reducing ends of hydrated bacterial cellulose (Evans et al., 2003). This approach has been shown to work with several additional metal complexes such as silver and gold, allowing us to elaborate electrically-conductive cellulose composites. We have also demonstrated a cellulose membrane modified with phosphate groups for ion-conductive membranes. For biomedical applications, we have investigated approaches for incorporating hydroxyapatite into the cellulose pellicles as bone replacement materials (Hutchens et al., 2006). Particulate materials, such as graphite powder, can be incorporated during culture of the bacteria creating an electrically conductive composite membrane for fuel cells (Evans, 2006). These examples highlight the versatility of bacterial cellulose for developing diversified composite materials for a wealth of applications.

Materials and Methods

Bacterial Strains

The taxonomic classification of the cellulose-synthesizing acetic acid bacteria is complicated by nomenclature issues. The cellulose-synthesizing bacteria that were used in these studies were originally classified as species of the genus *Acetobacter*. However, reclassification as a separate genus, *Gluconoacetobacter*, later shortened to *Gluconacetobacter*, had been purposed later (Yamada, 1997). As a result, either genus name may be employed for these bacterial strains depending on the authors or source. The names used by the American Type Culture Collection (ATCC), the source of the two strains used in these studies, are employed here.

Bacterial Cellulose Processing

Bacterial cellulose was purified as previously described, unless otherwise noted (Hutchens et al. 2006). For dry weight determinations of cellulose mass, bacterial cellulose samples were dried to thin paper-like membranes with a gel-dryer (Drygel Jr., Model SE540, Hoefer Scientific Instruments) for 20 minutes at 80°C under vacuum (20 inches Hg). The hydrated cellulose samples were placed on a layer of a hydrophilic synthetic fabric such as Miracloth (Calbiochem) or Pelon on the gel drier screen, then covered with a layer of Soloflo polyethylene fabric (Dupont). The dry samples were weighed with a Mettler AE200 electronic analytical balance (Mettler-Toledo: Toledo, OH, U.S.A).

Conductivity measurements

The conductivity of the media was measured using a Traceable Digital Conductivity Meter (Manufacturer's P/N 4063) (VWR International, Inc., Satellite Blvd., Suwanee, Georgia, 30024). MilliQ-purified water stored in a Pyrex glass bottle was used for washing the probe. Measurements were carried out on 20-ml aliquots of each calibration standard or media formulation in 50-ml polypropylene tubes. Two Traceable calibration standards, 100.6 μS/cm (9940 Ohms/cm) and 998 μS/cm (1002 Ohms/cm) consisting of KCl solutions in water were used for calibration of the meter. All measurements were carried out using the conductivity mode. Units were self-scaled by the instrument as μS/cm or mS/cm. When the reading mode was changed to megaohms/cm, the 100.6 S/cm standard gave a reading of 0.009 megaohms/cm, while the 998 S/cm standard gave a reading of 0.001 megaohms/cm.

Electric field alignment studies

Alternating electric fields (EFs) were generated using a Hewlett Packard function generator (model HP 8116A). Direct current EFs were generated using a Keithley SourceMeter 2602 or a Keithley 230 Programmable Voltage Source. Further experimental details are provided in the Results and Discussion section.

Ionic liquid studies

The 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)amide ([emim][Tf_2N])-based BC ionogel was prepared as outlined in **Figure 2**, noting that the cellulose pellicle employed was first cut to an approximately 47-mm diameter to match the diameter of a typical membrane filter prior to infusion with IL. The BC ionogel was dried overnight in a fume hood followed by storage in a vacuum dessicator for an additional day to degas the IL and fully remove residual water. The free-standing BC ionogel membrane was placed on a porous stainless steel support with a hydrophobic PTFE membrane with a 0.5 μm pore diameter added to prevent displacement of the IL onto the metal support. The membrane test system used has been previously described in detail (Mahurin, et al., 2010). Briefly, the system consists of permeate and retentate chambers separated by the membrane. Both chambers are initially pumped to vacuum prior to introducing the desired gas to the retentate chamber. The pressure rise in the permeate chamber was measured as a function of time to calculate the permeance, all permeance values being the result of measurement on a single gas.

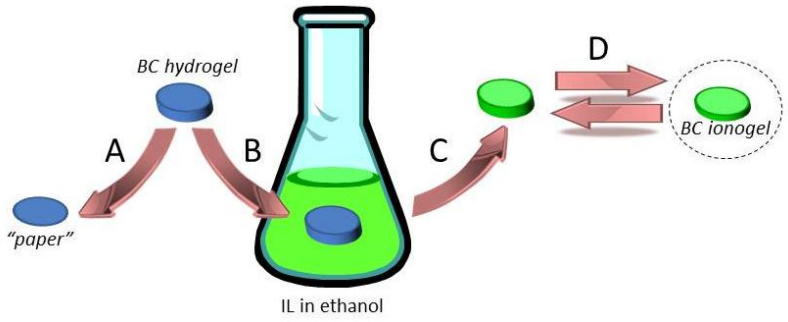

Figure 2. Cartoon schematic illustrating the solvent exchange process for preparing a BC ionogel, beginning with a BC hydrogel (A) The drying process for a BC pellicle occurs irreversibly, resulting in residual paper that cannot be rehydrated. (B) Conversely, by exchanging the water within the cellulose structure for an ethanolic solution containing IL, one can achieve a swollen BC pellicle analog, as shown by (C). Upon completion of drying, only the ethanol can be volatilized, resulting in an ionogel containing IL within a continuous BC network (D). This process is reversible, and the ionogel can be re-swollen in ethanol at will and the membrane thickness tuned by inclusion of a lesser or greater relative fraction (vol%) of IL.

Results and Discussion

1. Electric field alignment of bacterial cellulose during cultivation

1.1 Optimization of EF Medium for Cellulose-Synthesizing Bacteria

Initial attempts at EF alignment of bacterial cellulose for this project utilized a rich, undefined medium denoted as Schramm-Hestrin (SH) medium (Hestrin and Schramm, 1954). No clear evidence of alignment was apparent using this medium. A previous report of EF experiments with cellulose-synthesizing *Acetobacter xylinum* bacteria utilized a different medium, a high conductivity medium (Sano 2010), one using a synthetic salts formulation (Son 2003; **Appendix Table S1**) supplemented with corn steep liquor (concentration not stated), with fructose as the sugar. A reported study of the growth and cellulose production of *Acetobacter xylinus sucrofermentans* investigated the stimulatory effects of corn steep liquor and found that lactate was the ingredient that conferred the faster growth (Matsuoka 1996). The authors described mineral and vitamin supplements for preparation of a synthetic defined medium (Matsuoka 1996; Watanabe 2000).

Table 1. The different media formulations tested for optimization of *Gluconacetobacter* growth rate

Medium Number	1	2	3	4	5	6
0.04% sodium lactate	Y	Y	Y	Y	Y	N
0.5% soy peptone	Y	Y	Y	Y	Y	N
Son Basal Salts	Y	Y	Y	Y	N	Y
Matsuoka Vitamins	Y	Y	N	N	Y	Y
Matsuoka Minerals	Y	N	Y	N	Y	Y
4% Fructose	Y	Y	Y	Y	Y	Y

Media formulations were based on Matsuoka 1996, Son 2003 that describe corn steep liquor media and determination of lactate as the growth-promoting ingredient. Y and N indicate the presence and absence of a particular component, respectively.

An initial growth test (**Table 1 and Figure 3**) was carried out to determine the minimal medium additives required to obtain good cellulose formation using the two bacterial strains *Gluconacetobacter hansenii* (ATCC 10821) and *Acetobacter xylinus sucrofermentans* (ATCC 700178). The cellulose was purified and quantified, as described in Materials & Methods. Based on these tests, a formulation that did not require the addition of undefined components was devised and shown to produce fast growth and cellulose formation (**Table 2**). This formulation is named Son-Matsuoka-Fructose (SMF) and is derived from a combination of those described by Matsuoka and Son and contains 4% fructose and 0.04% sodium DL-lactate with vitamins and minerals.

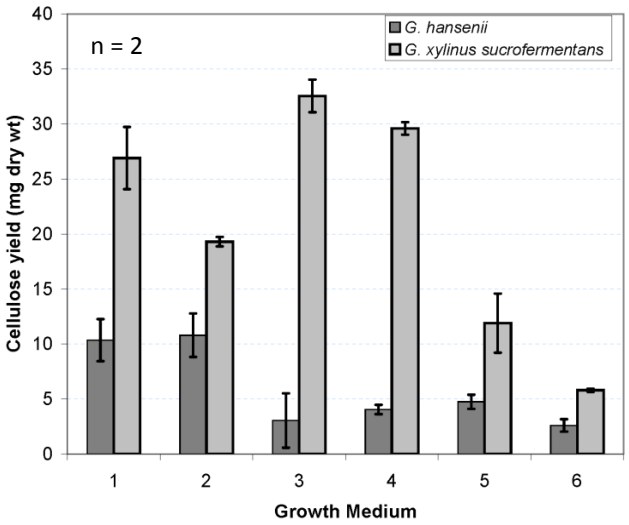

Figure 3. Cellulose yields for the two strains of bacteria grown in the six test media formulations described in Table 1. Data for two replicates are shown (n = 2)

Table 2. An optimized minimal salts high conductivity growth medium (named Son-Matsuoka-Fructose, SMF) based on the optimized components was developed for cultivation of *Gluconacetobacter xylinus sucrofermentans* for the EF experiments.

Basal Salts	**Concentration (%)**
$(NH_4)_2SO_4$	0.2
KH_2PO_4	0.3
$Na_2HPO_4 \cdot 12H_2O$	0.3
$MgSO_4 \cdot 7H_2O$	0.08
H_3BO_3	0.003
Carbon Sources	**Concentration (%)**
Fructose	4
Sodium DL-Lactate	0.04
Mineral Salts	**Concentration (mg/L)**
$FeSO_4 \cdot 7H_2O$	8.6
$CaCl_2 \cdot H_2O$	14.7
$Na_2MoO_4 \cdot 2H_2O$	2.4
$ZnSO_4 \cdot 7H_2O$	1.7
$MnSO_4 \cdot 5H_2O$	1.4
$CuSO_4 \cdot 5H_2O$	0.05
Vitamins	**Concentration (mg/L)**
Inositol	2
nicotinic acid	0.4
pyroxidine hydrochloride	0.4
thiamin hydrochloride	0.4
calcium pantothenate	0.2

Riboflavin	0.2
p-amino benzoic acid	0.2
folic acid	0.0002
D-biotin	0.0002

The conductivity of the media used was also determined because it is an important consideration for EF alignment studies. Three formulations of the undefined SH medium, with the 2% sugar being fructose, glucose, or sucrose, were measured. Three preparations of a high conductivity defined synthetic medium based on the recipes published by Son et al., 2003, and by Matsuoka et al., 1996 were also measured. This media formulation contains 4% fructose as the sugar and relatively high concentrations of ammonium sulfate (0.2%) and magnesium sulfate (0.08%). Both the SH and SMF media formulations had resistivity that was below the readable limit, so the ohms/cm values were calculated from the conductivity measurements. The SH media had a conductivity of 4.6 mS/cm regardless of the type of sugar, while the SMF media had a conductivity of 8.4 mS/cm (**Table 3**).

As part of the work we investigated the vital dye Acridine Orange (AO) as an approach to determine the effect of EF on cell viability. Both green fluorescence from live bacteria and orange fluorescence from AO complexed with RNA and DNA could be detected after 20 min incubation (**Figure S1**).

Table 3. Comparison of the conductivity and pH of the optimized SMF medium with SH media.

Medium	mS/cm	pH
SH Fructose	4.65	6.2
SH Glucose	4.58	6.2
SH Mannitol	4.68	6.2
SMF	8.40 ± 0.0212	5.513 ± 0.159

1.2 A modified culture apparatus for electric field alignment studies

In initial attempts to study the effect of EFs on the growth of the bacteria, different electrode materials and geometries, and also alternating current (AC) and direct current (DC) EFs were investigated. All cultures were grown in SH growth medium for 5-7 days. Cultures grown in AC EFs between 0.4 - 2 and V/cm at 0.1 – 10 kHz with aluminum (0.5 mm diam.) as the electrode material all produced cellulose that preferentially accumulated around the anode. However, the cellulose fibrils were not aligned as determined by scanning electron microscopy analysis. Similar results were also obtained when platinum was used as the electrode material. In the case of DC EFs with aluminum or platinum electrodes, cellulose formation was observed at 2V/cm or less. At higher voltages, ohmic heating and electrolysis of water were thought to be contributory factors in the inhibition cellulose growth. Similar to the results with AC EFs, no orientation of the cellulose fibrils was observed using SEM. In light of these results, it was decided to investigate approaches to optimize the conditions for cellulose production in EFs and also the geometry of the growth vessel for EF alignment experiments.

Indium tin oxide coated glass was used as the electrode material due to its good biocompatibility and low resistivity. The electrodes were placed in a plastic culture dish with inserts at regular intervals to allow different electrode spacings to be used (**Figure 4A**). This set-up produced a uniform EF in contrast to the EF produced by wire electrodes. In addition, the large surface area of the electrodes (2 x 5 cm) decreased ohmic heating effects during application of the EF.

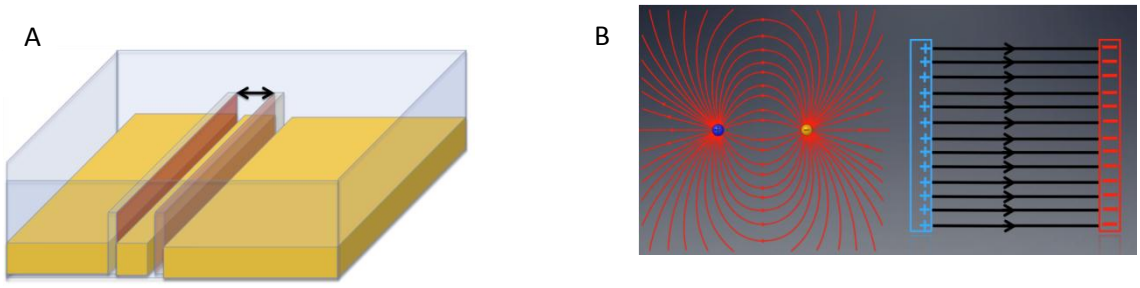

Figure 4. (A) Modified culture dish for electric field alignment studies. (B) Schematic representation of electric fields produced by wire electrodes (left) and planar electrodes (right)

1.3 Templated growth of bacteria for EF Experiments

Initial experiments examined the behavior of bacteria separated from the cellulose by physical methods then inoculated into fresh media. No clear directionality of cellulose formation could be identified following application of an EF to these, at least initially, free-floating bacteria.

Since these bacteria extrude the cellulose microfibrils as they move in the media, attaching the ends of the cellulose microfibrils to a defined point in the chamber at the start of cellulose synthesis would enable determination of any effect of the applied EF. The first attempt to establish a method to fix the starting point used agar patches. Bacteria were spotted onto agar patches in culture dishes. Following colony appearance, the culture dishes were filled with liquid media and incubation was continued. Strands of cellulose were synthesized that radiated out from the agar. However, the agar patches tended to loosen from the culture dish and the growth of the colonies on the agar patches was not uniform. Since the agar was necessarily on the bottom of the culture dish, the tendency of the bacteria to swim upward to the air-liquid interface could obscure an EF response.

Another strategy was developed to enable detection of response to an applied EF. Affinity of cellulose-synthesizing bacteria to surfaces coated with cotton cellulose and directional tracking of the bacteria along these surfaces had been reported (Kondo et al. 2002). The rapid attachment of the bacteria to pieces of plant cellulose, including fabric, and the subsequent synthesis of bacterial cellulose to completely encase the plant cellulose, had been noted previously (Evans and O'Neill, 2005).

Growth out from a string or thread suspended between the electrodes would provide a discrete, fixed starting point for the elongation of the bacterial cellulose during the EF experiments. Lengths of 6-ply cotton string or nylon core all-purpose mercerized thread (Coat's) were taped in 100-ml glass beakers such that a 4 cm length extended across the bottom of the beaker. The best results were obtained with thread. Sufficient SMF medium (10 ml) to cover the thread was added to each beaker, then 0.5 – 1.0 ml of a pre-culture of *A. x. sucrofermentans* grown in the same medium was added. The thread cultures were incubated on a rotary shaker at 120 rpm for 1 - 2 days to promote colonization of the thread by the bacteria. Uniform formation of bacterial cellulose along the submerged portion of the string or thread was observed. The colonized threads were placed between the two electrodes in the EF chambers and fastened in place using wooden sticks and tape. The distance between the electrodes was 1.8 cm for the plastic chambers and 2 cm for the glass chamber. The amperage of the EF chamber was monitored during several experiments. The amperage started at 50 – 200 μamp, then diminished over time to stabilize around 10-20 μamp (**Figure 5**). This indicates that the resistivity increased over time, suggesting that the electrode material becomes partially passivated with components from the growth media or the bacteria themselves. In one experiment, in which copper corrosion from the electrode connector poisoned the bacteria, the amperage level was observed to stay at 35 μamp and a deposit of Cu could be observed at the electrode surface. This provides further evidence for partial electrode passivation.

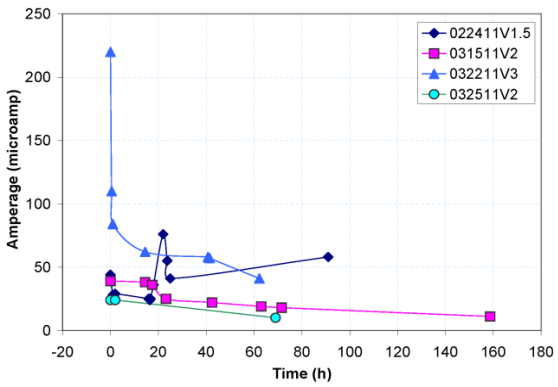

Figure 5. A consistent decrease in amperage was observed during the EF experiments, except for experiment 022411V1.5, in which copper ions leached from the electrode connectors inhibited bacterial growth.

Using thread pieces colonized with the bacteria, an apparent preference for growth in the direction of the anode was observed. Use of a glass slide box with a glass lid as one of the EF chambers enabled observation and photodocumentation of the bacterial cellulose synthesis during the experiments (**Figure 6**). Voltage was varied to determine the optimal levels for induction of a directional response. At 1.5 V applied voltage, there was not a clear preference for growth direction. The preference for the anode was greatest at 2 – 2.5 V (**Figure 7b and c**). Controls without applied voltage did not show a preferential growth direction (**Figure 7a**). Growth appeared to be decreased and the response was lessened at 3 - 3.5 V. These observations provide strong evidence for directional cellulose synthesis during application of the EF.

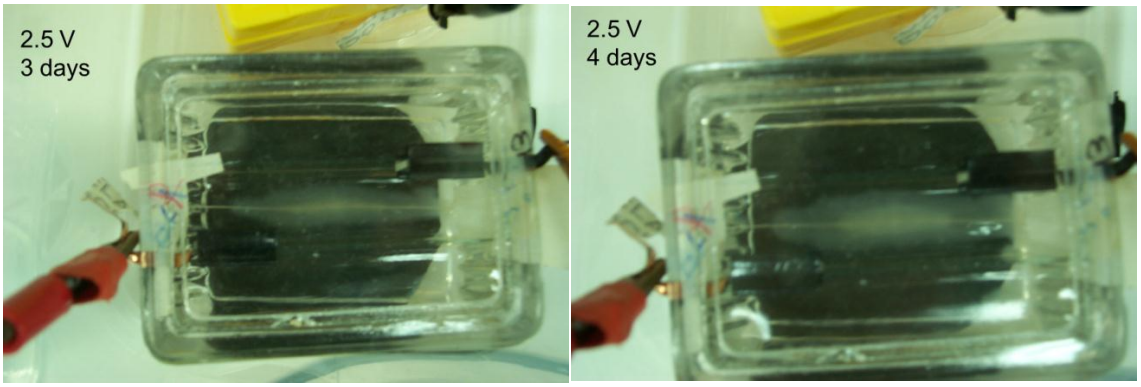

Figure 6. Bacterial cellulose from the bacteria adherent on a thread extended between the two electrodes preferentially grows toward the anode when a voltage of 2.5 V is applied across a 2-cm distance.

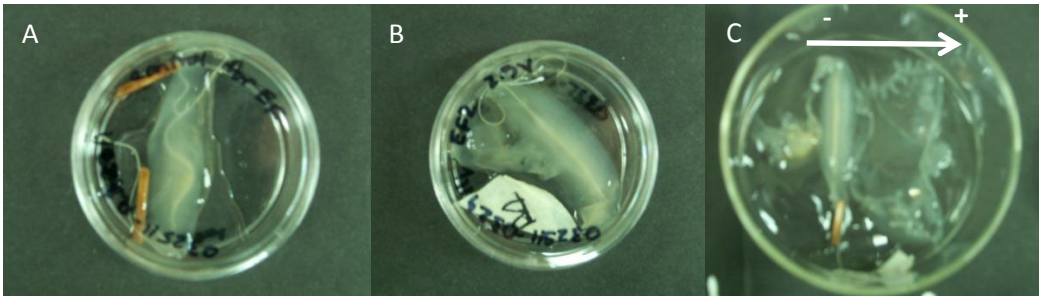

Figure 7. Bacterial cellulose samples were photographed following removal from EF chambers after 4 days growth. **(A)**, control grown without applied voltage; **(B)**, grown at 2.5 V and **(C)**, 2 cm electrode separation; bottom, grown at 2.0 V and 1.8 cm electrode separation.

1.4 Summary

The aim of this task was to devise a process for cellulose biosynthesis that would yield unidirectionally oriented nanofibers formed as fibrous cellulose sheets. Our preliminary studies showed that the choice of media formulation, the geometry of the apparatus, and the use of planktonic (free floating cell cultures) were all important issues for bacteria growth in EFs. In order to address these issues we devised three innovations: Firstly, a defined growth media was formulated (SMF medium) which had a higher conductivity than rich undefined media that are typically used for cellulose production. The combination of the high conductivity growth medium and an EF apparatus that had relatively large (2 x 5 cm) planer electrodes minimized ohmic-heating effects that can inhibit bacterial growth and also provided a uniform EF. However, the major innovation that we report is the idea of templated growth of the bacteria. This allowed the bacteria to be propagated from a fixed position in the EF chamber and provided unequivocal evidence for directional synthesis of cellulose during the application of an EF. Future work will require characterization of the cellulose fibers that comprise the sheets to

determine their alignment in the EF. In addition, approaches to produce larger cellulose mats in EFs will also be investigated.

2. Bacterial cellulose – ionic liquids composite materials

Ionic liquids (ILs) have various useful solvent attributes, being often noncorrosive, nonflammable, conductive, chemically inert, thermally stable, and vaporless under ordinary laboratory conditions. For example, conventional 1-alkyl-3-methylimidazolium ILs generally show no measurable vapor pressure below about 200 °C. By marrying the attractive structural features of bacterial cellulose (BC) with the outstanding liquid properties of ILs, BC ionogels were elaborated which proved to be amazingly robust and efficient membranes for gas separation applications, as illustrated by the high selectivity in the CO_2/N_2 separation problem.

2.1 Thermogravimetric analysis of composite material

Using the approach described in the Materials and Methods section, a BC ionogel containing an [emim][Tf_2N] mass fraction approaching that for the native hydrogel was achieved (~98 wt%). Cellulose ionogels formed flexible, durable, easy-to-handle, highly thermostable, vacuum-stable, and ionically-conductive membranes of tunable thickness (0.1–4 mm for the available pellicles). Pyrolysis of the BC component is evident in the derivative thermogram for the ionogel near 340 °C, accounting for only *ca.* 2% of the initial mass of the BC composite. BC ionogels are thermally stable to roughly 250 °C, as shown in **Figure 8**.

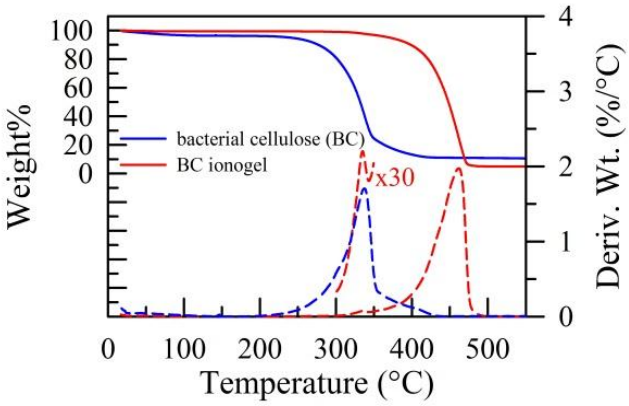

Figure 8. Scanning thermogravimetric profiles of native BC and a typical BC ionogel containing [emim][Tf_2N]. A heating rate of 10 °C min^{-1} was used.

2.2 BC Ionogel Gas Separation Results

After loading the BC ionogel membrane into the test system (see Mahurin, et al., 2010, for details), both chambers were pumped to vacuum and kept overnight to allow the membrane to equilibrate. Over the course of three days, the membrane remained within the test chamber

while the permeance was periodically measured. Multiple measurements of CO_2 and N_2 permeance were acquired and an average calculated. The CO_2 permeance was determined to be $1.39 \pm 0.05 \times 10^{-9}$ mol m^{-2} s^{-1} Pa^{-1} while the N_2 permeance was much lower ($4.4 \pm 0.3 \times 10^{-11}$ mol m^{-2} s^{-1} Pa^{-1}) giving a CO_2/N_2 selectivity of approximately 32 (**Figure 9**). These completely unoptimized values accord very favorably with previous results for the CO_2 and N_2 permeance values of 1.85×10^{-9} mol m^{-2} s^{-1} Pa^{-1} and 6.8×10^{-11} mol m^{-2} s^{-1} Pa^{-1}, respectively, measured using state-of-the-art commercial porous polyethersulfone membranes in liquid membrane CO_2/N_2 separations, employing the same IL. The slight differences that were observed can be due to the difference in the thicknesses of the two membranes.

After the initial three day test, the original BC ionogel membrane was placed on the shelf and left at ambient conditions for a full 70 days. After that time period, the membrane was re-loaded into the test system and degassed overnight. Again, the permeance of CO_2 and N_2 were measured yielding values of $1.8 \pm 0.05 \times 10^{-9}$ mol m^{-2} s^{-1} Pa^{-1} and $5.3 \pm 0.2 \times 10^{-11}$ mol m^{-2} s^{-1} Pa^{-1} were obtained, giving a CO_2/N_2 selectivity of approximately 35. These values are slightly higher than the originally measured values and are nearly identical to the values obtained using the polyethersulfone membrane support. This could result from minor losses of IL during the removal of the membrane after the initial three-day measurement cycle. In any case, these results clearly show that BC ionogels offer a unique framework to house ILs for gas separations as well as showing the promise of long-term stability for these hybrid membranes. Moreover, the permeance values for CO_2 and N_2 using the cellulosic support are in excellent agreement with values previously obtained using more traditional polymer membrane supports.

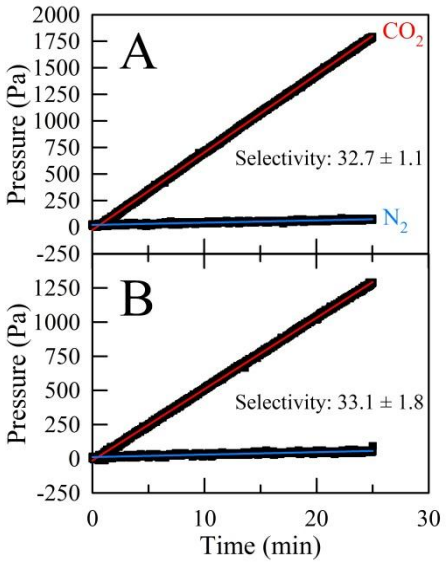

Figure 9. Representative single-gas permeation studies of CO_2 and N_2 through (A) [emim][Tf_2N] in polyethersulfone and (B) [emim][Tf_2N]-based BC ionogel liquid membranes.

2.3 Summary

Bacterial cellulose hydrogels are typically 99% water by mass. In this work we show that the water can be replaced by an ionic liquid, [emim][Tf_2N], without disruption of the structural properties of the cellulose matrix. Our data reveal that BC-derived ionogels perform comparably to, and in some respects better than, state-of-the-art commercial porous polyethersulfone membranes in liquid membrane CO_2/N_2 separations. This novel approach marries the functional properties of ionic liquids with the structural properties of bacterial cellulose for the development of a new class of membrane. We believe that these novel composites hold a wealth of potential beyond gas separations as well, including templates for producing functional materials, and in smart fabrics and sensory devices.

3. Carbonization of bacterial cellulose

Improvements in the carbon yield from bacterial cellulose will impact practical production of carbon fibers greatly. The maximum theoretical yield of carbon from cellulose ($(C_6H_{10}O_5)_n$) is 44.4 % if the hydrogen and oxygen are removed as water. However, typical carbon yields are only about 15% because volatile compounds such as methanol, acetic acid, carbon dioxide and tar substances are released (Kim et al 2001). In order to increase the yield of carbon from cellulose it is necessary to enhance dehydration during the course of pyrolysis. In this work we investigated different additives for their ability to enhance carbon yield during cellulose pyrolysis.

3.1 Sulfuric acid treated bacterial cellulose

Thermogravimetric analysis of native and treated cellulose is shown in **Figure 10**. The graphs are normalized to the starting weight of cellulose. The mass loss below 100^oC most probably represents removal of water from the samples. In addition, a higher yield of carbon is evident in the treated sample compared to native bacterial cellulose. The curves indicate that the pyrolysis of native bacterial cellulose is apparently a one-step reaction whereas the pyrolysis of treated cellulose proceeds in two-steps. This profile is similar to previously reported studies (Kim et al., 2001). On the other hand, the treated samples showed very different behavior. The curves indicate that the pyrolysis of pure bacterial cellulose is apparently a one-step reaction whereas the pyrolysis of treated cellulose proceeds in two-steps.

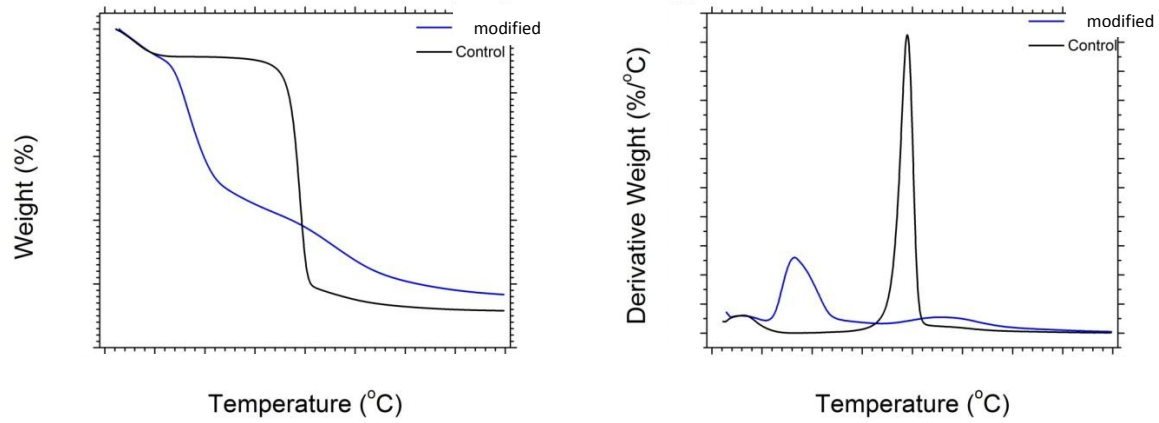

Figure 10. Thermogravimetric analysis of native and bacterial cellulose impregnated with a dehydrating agent

3.2 Pyrolysis studies

Purified bacterial cellulose was incubated with various concentrations of the dehydrating agent followed by pyrolysis under a inert gas mixture. A summary of the results for the dehydrating agent are presented in **Table 4** and demonstrate that up to 90% of the theoretical yield of carbon could be recovered. The carbonized cellulose material was then graphitized by heating under helium for several hours. The final yield of carbon was 59% of the theoretical yield, assuming complete retention of carbon from the cellulose (See **Table 4**)

Table 4. Comparison of carbon yield after carbonization of bacterial cellulose under different conditions

Sample	**Carbon yield (%)**
Cellulose	15
Cellulose	40
Cellulose	18
Cellulose	35
Graphitic Carbon	26
Theoretical C content	44

Figure 11 shows EDX spectra of carbonized cellulose under different conditions. The spectra show that the materials are nearly pure carbon with small amounts of oxygen present. This advantageous because it removes the need for downstream processing to remove impurities from carbon fibers.

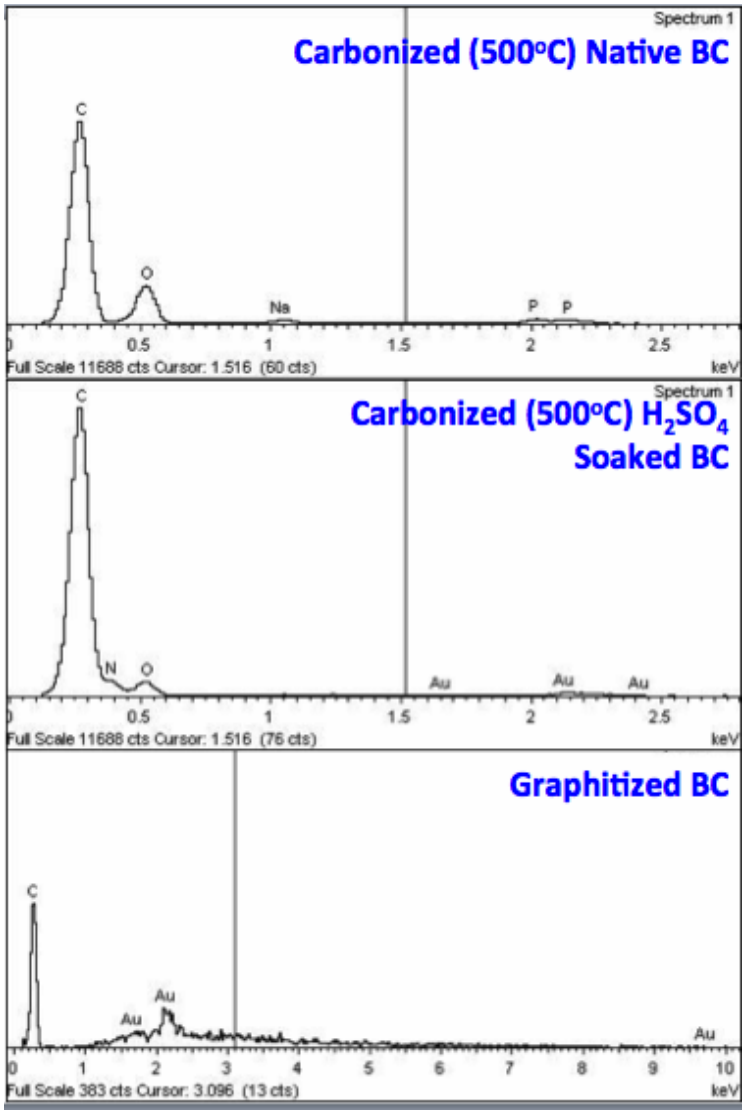

Figure 11. EDX analysis of carbonized cellulose samples

SEM analysis of the carbonized native bacterial cellulose, bacterial cellulose bacterial cellulose impregnated with a dehydrating agent after carbonization, and graphitized cellulose is shown in **Figure 12**. The fiber morphology was maintained during the carbonization process, even after graphitization which is advantageous in carbon manufacture from cellulosic materials

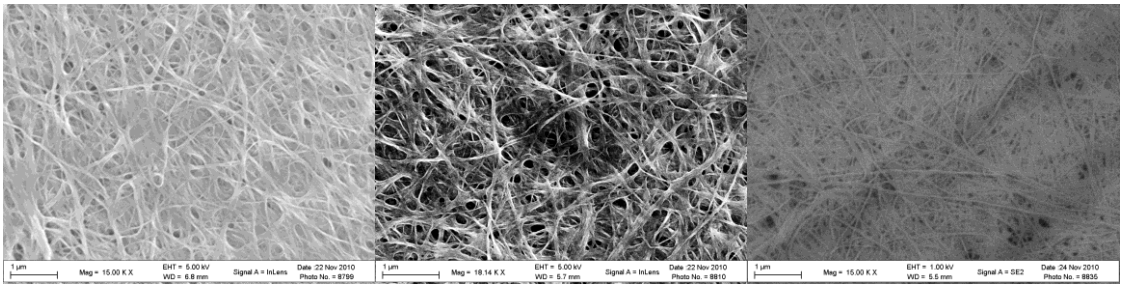

Figure 12. SEM images of carbonized bacterial cellulose: (A) native cellulose, (B) cellulose impregnated with a dehydrating agent, and (C) graphitized cellulose

3.3 Summary

This study shows that a near theoretical yield of carbon can be obtained from pyrolysis of bacterial cellulose at in the presence of a dehydrating agent. Thermogravimetric analysis revealed that the pyrolysis reaction occurred as a two-step process in contrast to pyrolysis of native cellulose which is a one-step reaction. It addresses concerns raised in previous studies on pyrolysis of plant cellulose that report low yield of carbon (~12-15%, see Kim et al., 2001 and references therein) and also questions raised during technical review of this proposal. This has important implications for economic production of high-value carbon fibers produced by EF alignment of cellulose.

Conclusions and Implications for Future Work

Our limited scope proposal provides critical proof-of-concept for developing new composite materials based on the renewable feedstock, bacterial cellulose, which reduce waste and do not contain hazardous materials. A major part of the work was to devise a process for cellulose biosynthesis that would yield unidirectionally oriented nanofibers formed as fibrous cellulose sheets. Our preliminary studies showed that the choice of media formulation, the geometry of the apparatus, and the use of planktonic (free floating cell cultures) were all important issues for bacteria growth in electric fields. By using a novel approach of templated growth of the bacteria to propagate the bacteria from a fixed position in the electric field chamber, provided unequivocal evidence for directional synthesis of cellulose during the application of an electric field.

Two different approaches for producing bacterial cellulose composite materials were also explored. The water in bacterial cellulose hydrogels was replaced with the ionic liquid, 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)amide, [emim][Tf_2N], and performed comparably to, and in some respects better than, state-of-the-art commercial porous polyethersulfone membranes in liquid membrane CO_2/N_2 separations. This novel approach marries the functional properties of ionic liquids with the structural properties of bacterial cellulose for the development of a new class of membraneFinally, an approach for carbonization of cellulose was devised and tested. By using a dehydrating agent it was possible to obtain a near theoretical yield of carbon after pyrolysis of bacterial cellulose, which has important implications for development of carbon-based composites and high-value carbon fibers from bacterial cellulose.

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Appendix

Table S1. The basal salts formulation described by Son 2003 was cited by Sano 2010 as the basis for a high-conductivity medium for *Acetobacter* that also contained corn steep liquor.

Component	Final Concentration (%)
$(NH_4)_2SO_4$	0.2
KH_2PO_4	0.3
$Na_2HPO_4.12H_2O$	0.3
$MgSO_4.6H_2O$	0.08
$FeSO_4.7H_2O$	0.0005
H_3BO_3	0.003

Fluorescent Staining of Cellulose-Synthesizing Bacteria

The vital dye Acridine Orange (AO) was used to stain the bacteria during cellulose synthesis by addition of a 100 X stock solution of 2 mg/ml filter-sterilized acridine orange in distilled water. Both green fluorescence from live bacteria and orange fluorescence from AO complexed with RNA and DNA could be detected after 20 min incubation (**Figure S1**). Samples stained with AO were fixed by cross-linking with 2% glutaraldehyde for 18 h. Following fixation and washing with sterile water, the fluorescence in the samples was stable for several weeks.

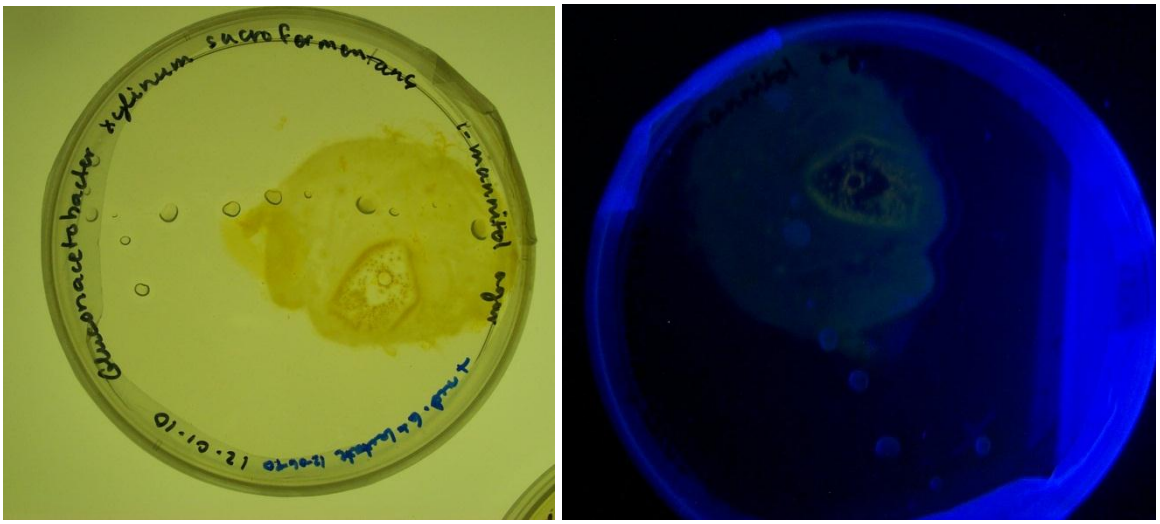

Figure S1. Visualization by illumination with white fluorescent light (left) and under a black lamp (8 W) emitting at 365 nm (right) of bacterial cellulose growth from colonies growing from an agar patch into liquid medium after staining with Acridine Orange